


Source of Genetic Aberrations in Human Embryonic Stem Cell: Common Fragile Sites and Replication Stress

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ABSTRACT

The capability of human embryonic stem cell (hESC) to form all cell types of the human body has made them highly attractive for therapeutic applications. Amongst others, the usefulness of hESC in therapeutic applications highly relies on their genomic integrity and stability. However, hESCs are well documented to frequently acquire genetic changes such as aneuploidies, segmental deletions or amplifications, epigenetic changes, and mitochondrial DNA mutations. This leads to safety concerns regarding the use of hESC in cell-based therapies. Certain genetic or epigenetic changes in hESC might lead not only to altered differentiation potential, but also increased proliferation capacity. A major concern is that, *in vivo,* this change might lead to tumorigenesis. These review will highlight the reported genetic aberrations found in human embryonic stem cell as a result of replication stress caused by naturally occurring common fragile sites in hESC.

Keywords: Common fragile sites, genetic aberrations, human embryonic stem cells, replication stress

ABSTRAK

Kemampuan sel punca embrionik manusia untuk berdiferensiasi membentuk seluruh lini sel tubuh manusia telah membuka peluang penggunaan sel punca untuk aplikasi terapetik. Penggunaan sel punca embrionik untuk aplikasi terapetik sangat bergantung pada stabilitas dan integritas genomiknya. Sayangnya, sel punca embrionik sering ditemukan memiliki perubahan-perubahan genetik seperti aneuploidi, duplikasi dan amplifikasi segmental, perubahan epigenetik dan mutasi DNA mitokondria. Hal ini menyebabkan munculnya isu keamanan penggunaan sel punca untuk terapi sel. Beberapa perubahan genetik ataupun epigenetik pada sel punca tidak hanya mengubah potensi diferensiasi namun juga kapasitas proliferasi sel sehingga menimbulkan kekuatiran transformasi sel menjadi sel kanker *in vivo*. Ulasan ini akan membahas kelainan-kelainan genetik yang ditemukan pada sel punca embrionik manusia yang disebabkan oleh stres replikasi oleh adanya situs rapuh umum. **Ivanna Williantarra. Sumber Kelainan Genetik pada Sel Punca Embrionik Manusia: Situs Rapuh Umum dan Stres Replikasi**

Kata kunci: Kelainan genetik,sel punca embrionik manusia, situs rapuh umum, stres replikasi

HUMAN EMBRYONIC STEM CELLS (HESC)

The billions of cells forming the human are all derived from one fertilized oocyte. After the zygote undergoes three cell divisions, the embryo contains eight identical cells. If one cell is removed at this stage, the other seven cells can still develop normally. The embryo at this point is therefore totipotent which means it can develop to all human cell types. Veiga[1] showed that 4-cells stage blastomeres to be also 'potentially totipotent', this was further confirmed by Van de Velde.[2] After compaction, the embryo develops to the blastocyst stage. Blastocysts consist of trophectoderm and inner cell mass (ICM) cells. The trophectoderm gives rise to the umbilical cord and placenta whereas the ICM gives rise to the fetus. The ICM is pluripotent at this stage. This pluripotent state lasts only shortly, and the development and differentiation processes follow quickly.[3,4]

Stem cells are cells which can give rise to themselves and at least one other more specialized (differentiated) cell type. Adult stem cells are found in postnatal tissues and are either multipotent, which means the spectrum of the cells they can form is limited to those normally present in the organ from which they are derived, or unipotent, which means they can only divide and develop to one cell type. Embryonic stem cells are derived from a pre-implantation embryo and in the human are considered totipotent, which means they can form all cell types of the body and can form extra-embryonic tissues.

ANEUPLOIDIES AND STRUCTURAL VARIATIONS IN HESC

Chromosomal abnormalities in hESC have been repeatedly reported. According to Nguyen,[5] aneuploidy has been reported to all chromosomes at least once. A study by Amps[6] revealed that cultured hESC most frequently acquire gains of entire or part of chromosomes 1, 12, 17 and 20. Additional copies of chromosome X have also been observed.[7] The most commonly found mutation in hESC is the gain of 20q11.21.[8,9] Remarkably, chromosomal deletion is much less frequent than chromosomal gain in hESC.

The high frequencies of chromosomal gain in chromosome 1, 12, 17, 20 and X indicate that these chromosomes might contain genes

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essential for cell growth or survival.[10] A study by Nguyen[5] showed that gain of 20q11.21 caused an overexpression of Bcl-xL leading to apoptosis-resistant cells. Non-random gain of chromosome 17 has also been reported in several cancer cases, indicating that this chromosome might house genes involved in malignant transformation.[11] One of those genes that might play a role in malignancy transformation after duplication is *BIRC5*, an anti-apoptotic gene located on chromosome 17.[12] Another important gene is *NANOG* which is located on chromosome 12. *NANOG* plays a very important role in maintaining pluripotency and the prevention of differentiation, so that overexpression of *NANOG* confers a selective advantage to hESC.[13,14] Other genes of interest located on chromosome 12 are *KRAS* and *SOX5*. Amplification of *KRAS* and *SOX5* reduces apoptosis, a hallmark characteristic of cancer.[15]

The appearance of de novo structural variations in cells can be caused by several factors, a very important being replication stress, due to conditions that challenge replication fork progression.

REPLICATION STRESS

DNA Damage Repair

Zeman[16] defined replication stress as the slowing or stalling of replication forks, but replication stress does not always lead to replication defects. Single stranded DNA (ssDNA) is commonly found as a result of replication stress. ssDNA will attract replication stress response proteins including kinase ataxia telangiectasia mutated (ATM) and Rad3 related (ATR). These two kinases will phosphorylate downstream proteins which help cell survival and ensure replication completion by activating cell cycle checkpoints and inhibition of firing of late replication origins.[17] DNA damage checkpoint can cause G1/S arrest to prevent damaged DNA replication, G2/M arrest to prevent segregation of damaged DNA during mitosis, or S phase responses to ensure replication of entire genome before the cell divides.[18] Generally, checkpoint control will block the start of DNA replication at other replication origins and entry to mitosis phase while DNA repair systems are promoted. The checkpoint proteins involve three major groups of proteins including (a) sensor proteins which detect damaged DNA and initiate the cascade reaction; (b) transducer proteins which amplify the signal and phosphorylate themselves or other downstream target kinases and (c) effector proteins which are the most downstream targets of the cascade and prevent cell cycle progression and repair the DNA.[19] ATR stabilizes the replication fork until the source of stress is removed. Once the replication stress is removed, the forks can be restarted.[20] The kinase activity of ATR has generated several markers which can be used to detect replication stress, including the H2AX (γH2AX) histone variant as shown in figure 1, RPA and CHK1 phosphorylation.[16]

There are two major repair pathways; homologous recombination (HR) and non-homologous ends joining (NHEJ). Broken ends (in contrast to the normal telomeric end) are very reactive; they interact with high frequency with other broken ends (NHEJ) or with the homologous sequence on the non-identical sister chromatid (HR). HR is the major repair pathway for DNA double stranded breaks which are generated during replication, and since the DNA repair is dependent on an undamaged template it tends to be less error-prone than NHEJ. While NHEJ is active throughout the cell cycle, the necessity of presence of a sister chromatid makes HR only active during S and G2 phase when DNA replication has finished.[21,22]

Maintaining the genome integrity is essential for stem cells because all progeny, including the differentiated derivatives, will inherit the mutations. Stem cells have a higher level of DNA repair proteins expression and show greater repair capacity.[23] The first event that occurs after DNA breakage is the phosphorylation of H2AX. This process is mediated by ATM, ATR or DNA-PKcs. In hESC, the number of γH2AX foci is larger than in somatic cells and the main kinase mediating this is the ATR instead of ATM in somatic cells. Inhibition of ATR and ATM is very toxic for hESC. ES cells have shorter G1 phase compared to somatic cells and therefore the DNA repair system is dominated by HR. Despite the short G1 phase, the G2 phase is very long in hESC.[24]

Sources of Replication Stress

Presence of nicks, gaps and ssDNA, and misincorporation of ribonucleotides are major causes of replication stress. However, some DNA regions are intrinsically challenging for replication machinery. These regions are called the fragile sites. Fragile sites (FS) are nonrandom loci in chromosomes which exhibit increased frequency of DNA breakage under replication stress.[25] CFSs (Common Fragile Sites) are considered the preferential target of genomic instability and mutations in CFSs are often found in cancers even from its early progression.

CFS instability is caused by multiple factors such as its intrinsic properties and interference of the replication process, as shown in figure 2. CFSs contain repeats which cause them to have the tendency to readily fold to secondary structures. Formation of secondary structures will increase CFS proneness to fork stalling or perturbation of strand elongation during the replication process. It has also been proposed recently that CFS might also be caused by the transcription process. Many CFSs have been mapped to be located on the coding region of large human genes. The large genes require longer time to be transcribed causing higher possibility of transcription machinery and replication forks to collide forming a rare R-loop structure. DNA polymerase will inhibit the elongating RNA polymerase and stable R-loops are created at the site of blockage, contributing to breakage at CFS. Moreover, the relative lack of origins of replication also leads to a higher possibility of defects during the replication process due to depletion of nucleotide pools.[16,25-27]

Another remarkable fact is that CFS expression is cell type dependent which indicates that CFS instability is determined epigenetically, affecting the fork origin and timing.[26,28] Epigenetic changes, such as increased numbers of chromatin loops and histone acetylation will reduce the numbers of replication origins and thus lead to increased expression of CFSs.[29] Jiang[30] suggested that DSBs are more frequently found in heterochromatic region.

Factors Affecting Genetic Aberrations in hESC

Several factors have been reported as the source of genetic aberrations in hESC. The first possible source is the quality of the embryo from which the cell line is derived.[31,32] Some embryos were donated to research because of their poor quality and some were donated because of their genetic abnormalities.[31] In the case of acquired abnormalities, it has been


suggested that their incidence correlates to the time the hESC are kept in culture (number of passages) and passaging technique.[32] Passaging techniques that expose cells to less selective pressure, such as manual passaging compared to single cell dissociation, may reduce the acquisition of genetic aberrations.[33] Freezing and thawing are other pressure points which may influence the chromosomal stability of the cell lines.[7]

Jacobs[34] has identified culture density as an important driver of genome instability in hESC. In his study, he found that hESCs that are cultured in higher density show a higher degree of DNA damage and chromosomal abnormalities. Denser cultures expose the cells to an accumulation of metabolism products in the medium. These cause replication stress to the cells, which leads to replication fork stalling and formation of DNA breaks. Culture medium analysis showed that denser cultures lead to high concentrations of lactic acid, which result in medium acidification. He proved that the DNA damage was indeed caused by the medium acidification by lactic acid by adding lactate to low density cultures.

Refreshing the medium more often proved to significantly decrease the metabolite accumulation, rescuing the DNA fragmentation to background levels. It is therefore suspected that by controlling the medium, the DNA damage can be minimized in denser hESC cultures. Moreover, if DNA damage is caused by medium acidification, medium with a wider buffer range will have benefits to maintain DNA integrity in hESC culture. Also, other culture systems might reduce the influence of the acid on the cells, or cause metabolic changes so that less lactic acid is produced.

CONCLUSION

The high frequencies of genetic aberrations repeatedly reported in human embryonic stem cells have hurdled further application of stem cell in cell therapy. Aneuploidies and other structural variations observed in human embryonic stem cells might be caused by the intrinsic properties of the genome which leads to replication stress. Many factors have been identified to cause genetic aberrations in human embryonic stem cells such as cell density, culturing system and passaging techniques. Methods to ensure the genetic stability of cultured stem cell thus need to be further researched. The necessity of culturing systems along with the observed effects of mediums and cell densities for genetic stability of hESC have prompted researchers to finding other culture systems which are more suitable to sustain genomic integrity of hESC. Further work is necessary to understand the factors that influence how cells react to genotoxic stress, and the reasons for these differences.

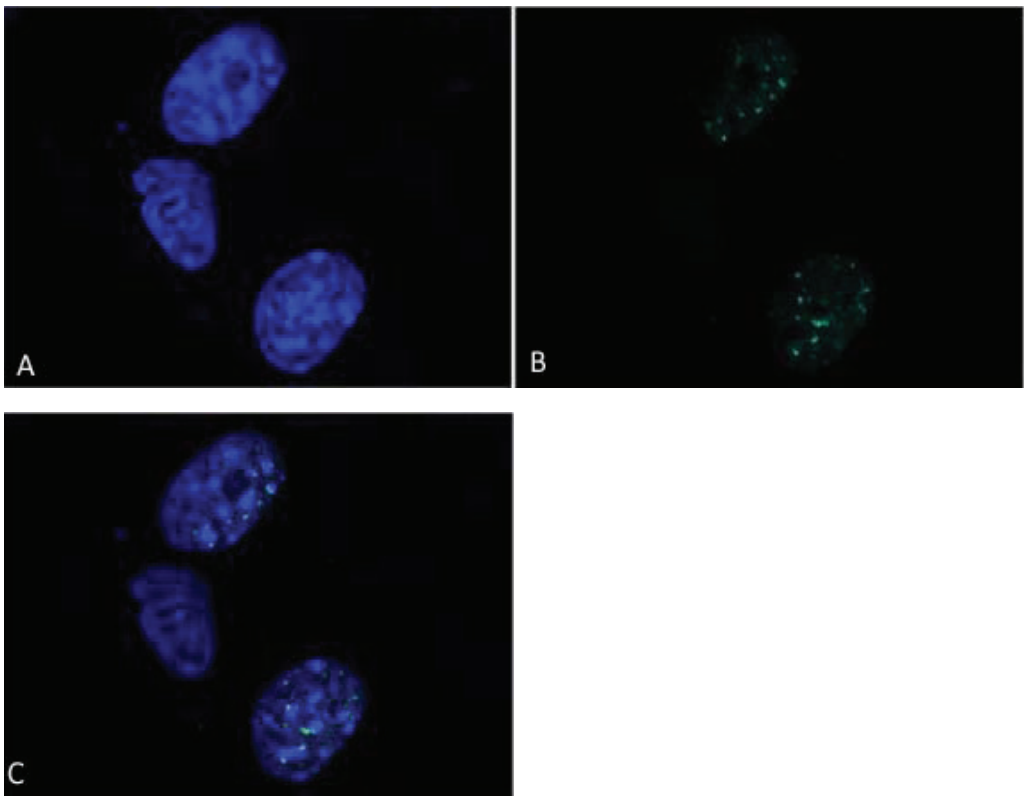

Figure 1. Double strand breaks staining using antibody to detect phosphorylated serine 139 of histone H2AX as a marker (96x magnification). The DAPI nuclear staining shows the nucleus in blue (A) and the green dots inside the nucleus were phosphorylated H2AX (B). Figure C shows the merged image.

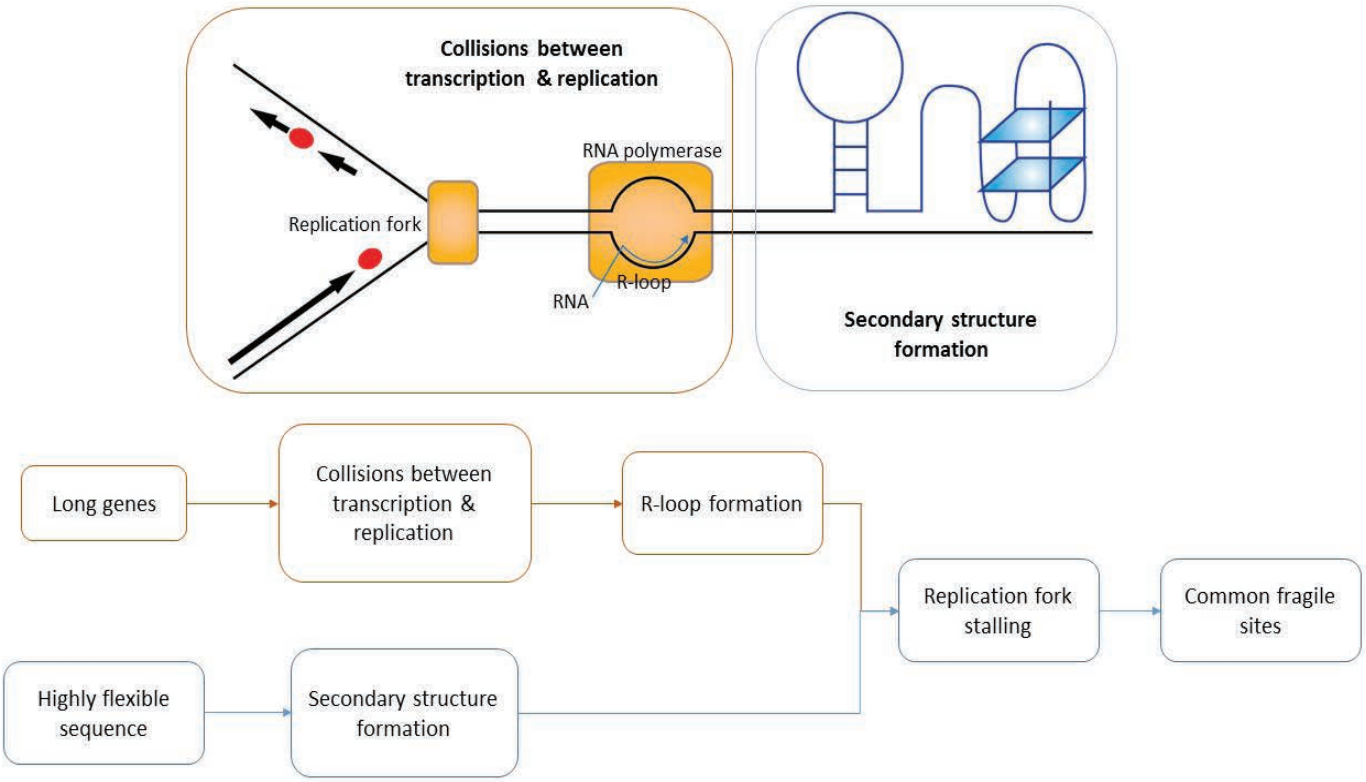

Figure 2. Potential factors contributing to CFS fragility. CFS fragility is affected by both intrinsic and extrinsic characteristics of CFS. The intrinsic factors include tendency of CFS sequence to form secondary structures and the scarcity of replication origins within CFS. Extrinsic factors involve transcription process of long gene which will form a rare R-loop structure. Collisions between transcription and replication is an example of replication fork stalling due to both intrinsic and extrinsic factors.

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